

Adipocytes Secrete Leukotrienes

Contribution to Obesity-Associated Inflammation and Insulin Resistance in Mice

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Leukotrienes (LTs) are potent proinflammatory mediators, and many important aspects of innate and adaptive immune responses are regulated by LTs. Key members of the LT synthesis pathway are overexpressed in adipose tissue (AT) during obesity, resulting in increased LT levels in this tissue. We observed that several mouse adipocyte cell lines and primary adipocytes from mice and humans both can secrete large amounts of LTs. Furthermore, this production increases with a high-fat diet (HFD) and positively correlates with adipocyte size. LTs produced by adipocytes play an important role in attracting macrophages and T cells in *in vitro* chemotaxis assays. Mice that are deficient for the enzyme 5-lipoxygenase (5-LO), and therefore lack LTs, exhibit a decrease in HFD-induced AT macrophage and T-cell infiltration and are partially protected from HFD-induced insulin resistance. Similarly, treatment of HFD-fed wild-type mice with the 5-LO inhibitor Zileuton also results in a reduction of AT macrophages and T cells, accompanied by a decrease in insulin resistance. Together, these findings suggest that LTs represent a novel target in the prevention or treatment of obesity-associated inflammation and insulin resistance. *Diabetes* 61:2311–2319, 2012

Obesity is associated with a state of chronic, low-grade inflammation that contributes to insulin resistance (IR), type 2 diabetes, and increased risk for cardiovascular diseases. In both humans and rodents, inflammatory cells accumulate in adipose tissue (AT) with increasing body weight, and evidence is mounting that implicates these inflammatory cells as significant contributors to obesity-associated IR (1). More specifically, obesity leads in AT to a shift in balance of anti-inflammatory M2 macrophages and T-helper 2 (Th2) and regulatory T cells (Tregs) toward proinflammatory Th1 cells and an influx of cluster of differentiation 8 (CD8)⁺ effector T cells, subsequently resulting in the recruitment and differentiation of proinflammatory M1 macrophages (1). The

resulting increase in the production and secretion of proinflammatory factors leads to IR and type 2 diabetes.

An active area of current research focuses on identifying the trigger(s) driving the recruitment of inflammatory cells to obese AT. This study focuses on the potential role of leukotrienes (LTs) in obesity-associated inflammation and IR.

LTs are potent proinflammatory mediators, and many important aspects of innate and adaptive immune responses are regulated by LTs (2). LTB₄ is a potent leukocyte chemoattractant and activator (2). This LT promotes the generation of M1 macrophages (3). However, cysteinyl-containing LTs (CysLTs; LTC₄, -D₄, and -E₄) contract smooth muscles, particularly in the peripheral airways, and are regarded as pivotal mediators of bronchial asthma (2). LTB₄ and CysLTs are potent chemoattractants for T cells (4–6). Furthermore, LTB₄ inhibits Treg differentiation and stimulates T_H17 differentiation, a T-cell subset recently shown to be increased in obesity (7,8).

Several studies have suggested a potential link between the LT pathway and AT inflammation (9). The expression of the enzyme 5-lipoxygenase (5-LO) and its nonenzymatic cofactor 5-LO activating protein (FLAP) were both increased in obese AT (10,11). These major players in LT biosynthesis were expressed in adipocytes and in AT macrophages (ATMs) in obese AT (10,11). A recent study demonstrated that AT from obese mice, compared with AT from lean mice, exhibited increased LTB₄ levels. Furthermore, FLAP inhibition resulted in decreased ATM infiltration and improvement of insulin sensitivity (12). Lastly, while the manuscript of this article was being prepared, a study was published showing that mice lacking the LTB₄ receptor BLT1 exhibit a similar decrease in ATM infiltration and improvement of insulin sensitivity in a model of diet-induced obesity (13).

In our work, we analyzed the production of LTs by adipocytes and their role in *in vitro* chemotaxis assays for macrophages and T cells. We also studied the role of LTs in AT infiltration with macrophages and T cells, and the subsequent development of IR, in mice deficient for 5-LO or treated with its inhibitor Zileuton.

RESEARCH DESIGN AND METHODS

Animal studies. All experimental procedures were conducted according to French legislation. Breeder pairs for 5-LO^{-/-} mice (B6.129S2-Alox5tm1Fun/J), which were backcrossed nine times to a C57BL/6J background, were purchased from The Jackson Laboratory and were subsequently bred in-house to yield the 5-LO^{-/-} male mice used in this study. Male C57BL/6J mice used as controls and for the Zileuton-treatment studies were also purchased from The Jackson Laboratory. Both groups were fed a normal chow (NC) diet (5.1% kcal from fat; UAR A03, Villemoisson, France) throughout the study or were started on a high-fat diet (HFD; 60% kcal from fat; D12492, Research Diets) at age 12

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weeks for up to 15 (5-LO^{-/-} studies) or 17 weeks (Zileuton studies). Weight gain and food intake were monitored throughout the diet.

Glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) were performed as described previously (14). Insulin was measured using the Ultra Sensitive Rat Insulin ELISA kit (Crystal Chem). Weight-matched studies were achieved by performing metabolic testing 2–3 weeks earlier on the 5-LO^{-/-} compared with the wild-type (WT) mice, whereas diet duration-matched studies were performed at the same time on both genotypes. For the Zileuton studies, HFD-fed mice received oral Zileuton (100 mg/kg; Tocris Bioscience) or vehicle control (10% [v/v] DMSO in normal saline) daily during a 2-week period. Primary adipocytes and stromal vascular cells (SVCs) were isolated from AT, as described previously (14).

Human studies. The AT samples were collected from individuals undergoing elective surgical procedures of fat removal for aesthetic purpose. Informed consent, concerning the use of the AT samples for research only, was asked before plastic surgery. Samples were collected in the research laboratory without any data concerning the identity of the donor except sex, age, and BMI. The samples used for this study all came from women with an average age of 42 years (range 33–57) and an average BMI of 28 kg/m² (range 21–38).

Mature adipocytes were isolated from subcutaneous white AT (scWAT), as previously described (15). An aliquot of adipocytes was fixed (BD CellFIX solution, BD Biosciences, France), and the number and diameter (average, 200 cells) were determined under a microscope (Nikon). Adipocytes (10⁷/mL) were maintained for 30 min at 37°C in Krebs-Ringer-bicarbonate buffer (pH 7.4) containing 0.1% (w/v) fatty acid-free, low endotoxin BSA for LT production.

Real-time quantitative PCR analysis. Total RNA was extracted from AT with Trizol reagent following the supplier's protocol (Invitrogen). Total RNA (0.5 µg) was reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen). Quantitative PCR (qPCR) was done using Power SYBR Green PCR master mix (Applied Biosystems) on an ABI Prism 7000 Sequence Detector System (Applied Biosystems). The mRNA levels of all genes reported were normalized to cyclophilin A transcript levels. Primer sequences are available upon request.

Eicosanoid extractions and measurements. Eicosanoids were extracted from AT samples (200 mg) using 6-mL solid-phase extraction cartridges (C18, Cayman Europe) following the manufacturer's instructions. LTB₄ and CysLT levels in tissue extracts and conditioned media (CM) were simultaneously measured using a Luminex 200 machine (Merck Millipore) by multiplexing a Luminex LTB₄ with a compatible CysLT kit (Cayman Europe). The AT samples from 5-LO^{-/-} mice were used as negative controls and were spiked with LTB₄ and CysLTs (50 pg for both) before LT extraction to measure recovery. We measured LT concentrations at background levels in these negative control samples and had on average 89% recovery of LTs extracted from the spiked samples (data not shown). The AT LT levels reported were corrected accordingly and normalized for cell numbers that were estimated based on DNA concentrations measured using a Quant-iT PicoGreen dsDNA kit (Invitrogen).

Cell culture studies. 3T3-L1 mouse embryo fibroblasts were obtained from the American Type Culture Collection. 3T3-F442A cells were a gift from H. Green (16), and the Ob17 cell line was established in our laboratory (17). These cells were cultured and differentiated into mature adipocytes as described previously (16). Where indicated, mature 3T3-L1 adipocytes underwent a 24-h treatment with 10 ng/mL lipopolysaccharide (LPS) or CM obtained from RAW264.7 macrophages. For the Zileuton studies, 3T3-L1 mature adipocytes (day 8–11) were washed twice with PBS and then incubated for 24 h in serum-free DMEM with 0.2% BSA containing vehicle (1% [v/v] DMSO) or with increasing concentrations of Zileuton. Cells were washed and underwent a second 24-h period of incubation with drug- and serum-free media before CM harvest. The adipocyte CM was centrifuged and filtered before use.

Chemotaxis assays. RAW264.7 macrophages were donated by G. Carle and Jurkat T cells by J.-E. Ricci (both from University of Nice-Sophia Antipolis, France), and these cell cultures were maintained in DMEM or RPMI-1640 supplemented with 10% (v/v) FBS, respectively. Chemotaxis assays were essentially performed as described previously for RAW264.7 macrophages (18), with the exception that migration of Jurkat T cells (grown in suspension) was quantified by counting the cells found in the lower chamber using a hemocytometer. Results were presented as the chemotactic index or ratio of cells in the lower chamber in the presence versus the absence of chemoattractant (serum-free DMEM).

Cytokine measurements. Cytokine/chemokine measurements in mouse serum and adipocyte CM were performed using two different five-plex multiplex assays adapted to serum or tissue culture media (Merck Millipore) on a Luminex 200 machine (Merck Millipore).

Immunohistochemistry and quantification of crown-like structures. Macrophages were stained, followed by quantification of crown-like structures, as described previously (14). Essentially, the same procedures were followed for T-cell stainings using a CD3 (ab5690, Abcam) antibody and an anti-rabbit secondary antibody (Vector Laboratories), both at a 1:50 dilution.

Fluorescence-activated cell sorter (FACS) analysis. SVCs were incubated with Fc Block (BD Biosciences, San Jose, CA) for 20 min at 4°C before staining with fluorescently labeled primary antibodies or control IgGs for 25 min at 4°C. F4/80-allophycocyanin, CD11b-phycoerythrin-Cy7, CD11c-phycoerythrin, CD3-phycoerythrin, CD4-fluorescein isothiocyanate, and CD8-allophycocyanin FACS antibodies were purchased from eBioscience. Cells were gently washed twice and resuspended in FACS buffer with DAPI. SVCs were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Unstained, single stains, and FMOs (fluorescence minus one) controls were used for setting compensation and gates (see Supplementary Fig. 1 and Fig. 2).

Statistical analysis. All calculations were performed using Prism 3.02 software (GraphPad, San Diego, CA). Statistical significance between two groups was determined by the Student *t* test. Comparisons among several groups were performed by ANOVA. When the results passed the ANOVA test, we performed Bonferroni multiple comparison post-tests to calculate the relevant *P* values.

RESULTS

LT production in AT and adipocytes. To analyze the effects of anatomic location and diet on AT LT production, we measured LTB₄ and CysLTs, in sc-WAT and epididymal WAT (epi-WAT) from NC- and HFD-fed mice. Figure 1A shows that LTB₄ levels are three- to fourfold higher in epi-versus sc-WAT and increase another three- to fourfold with a HFD. Figure 1B shows similar results for CysLTs, although the depot- or diet-dependent changes in CysLT levels amount to only about a 30–40% increase. Overall, LTB₄ levels are approximately 10- to 20-fold higher than CysLT levels.

When measuring LT secretion by 3T3-L1 adipocytes, we discovered that adipocytes secrete large amounts of LTs (Fig. 1C) compared with RAW264.7 macrophages, which have been described to secrete LTs (19). To verify whether this was not just an artifact of the 3T3-L1 cell line, we also measured LT secretion by two other adipocyte cell lines (Fig. 1C). 3T3-F442A and Ob17 adipocytes secrete LTB₄ and CysLTs in differing amounts for each cell line. We could not detect LT secretion by 3T3-L1 preadipocytes. This is in agreement with relatively low mRNA levels of LT synthesis pathway enzymes in preadipocytes versus mature adipocytes (Supplementary Fig. 3). LT production by 3T3-L1 adipocytes was increased after treatment with LPS or RAW264.7 macrophage CM, suggesting that inflammatory stimuli increase LT production in adipocytes (Supplementary Fig. 4).

In addition to these adipocyte cell lines, we measured LT secretion by primary mature adipocytes and SVCs isolated from epi-WAT from NC- and HFD-fed mice (Fig. 1D and E). Figure 1D shows that primary mature adipocytes derived from epi-WAT secrete LTB₄ and CysLTs and that production increases with HFD-feeding. LT secretion by primary mouse mature adipocytes is higher than in mouse adipocyte cell lines. SVCs also secrete LTs in a similar diet-dependent manner but secrete more LTB₄ and CysLTs than primary adipocytes (Fig. 1E). We adjusted for the proportions of both cell subsets in AT (see Supplementary Fig. 5) to calculate the relative contribution of each subset to the total LT production by AT (Fig. 1F). This demonstrates that adipocytes account for 45% of the total LTB₄ and 24% of the total CysLT production in AT from mice fed NC. With the HFD, this contribution decreases to 33% and 12%, respectively, due to a reduction of adipocytes per gram of AT (most likely due to increased adipocyte size) and a slight (nonsignificant) increase of SVCs (perhaps due to infiltration of inflammatory cells).

We also measured LT secretion by human primary mature adipocytes isolated from sc-WAT. We observed that

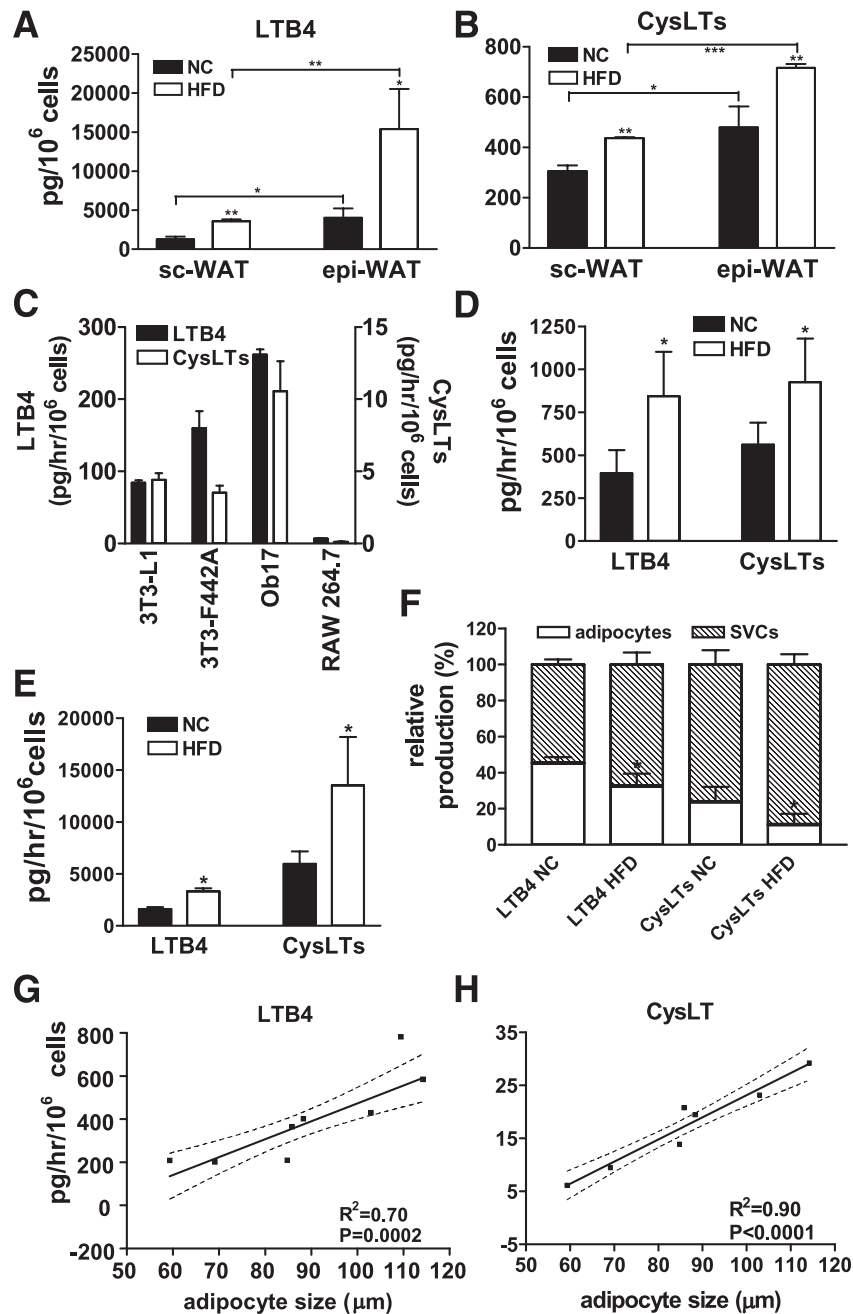


FIG. 1. LT production in adipose tissue and adipocytes. LTB4 (A) and CysLT (B) levels in pg/million cells in sc- and epi-WAT derived from NC- or HFD-fed mice ($n = 6$). C: LTB4 (left y-axis) and CysLT (right y-axis) secretion in pg/h/million cells by 3T3-L1, 3T3-F442A, or Ob17 adipocytes compared with RAW264.7 macrophages. LTB4 and CysLT secretion in pg/h/million cells by primary adipocytes (D) or SVCs (E) isolated from epi-WAT derived from NC- or HFD-fed mice ($n = 4$ /group). F: Relative contribution in percentage of adipocytes and SVCs to total epi-WAT LT production. LTB4 (G) and CysLT (H) in pg/h/million cells secreted by primary adipocytes isolated from human sc-WAT in correlation with adipocyte size ($n = 8$). Black squares represent the data points used to perform linear regression analysis (solid line). Dashed lines represent the 95% confidence interval for the regression line. Data are expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

human primary mature adipocytes also secrete LTs and that their levels positively correlate with adipocyte size (Fig. 1G and H). There was only a mild ($R^2 = 0.506$, $P = 0.0479$) or no correlation ($R^2 = 0.0072$, $P = 0.8352$) between BMI and LTB4 and CysLT secretion, respectively (data not shown).

Role of LTs in adipocyte-induced RAW264.7 macrophage and Jurkat T-cell chemotaxis and adipokine secretion.

LTs are known to be potent macrophage and T-cell chemoattractants (2,4–6). Because we demonstrated that primary adipocytes can secrete LTs in a diet-dependent manner

(Fig. 1), we tested the macrophage chemotactic potential of CM obtained from primary adipocytes derived from epi-WAT from NC- and HFD-fed WT mice and compared that with that of HFD-fed LT-deficient 5-LO^{-/-} mice (Supplementary Fig. 6). These results demonstrate that primary adipocytes isolated from obese AT secrete more chemotactic activity than those isolated from normal AT and that this increase is lacking in the absence of LTs. To study this further, we measured RAW264.7 macrophage and Jurkat T-cell chemotaxis stimulated by CM harvested from 3T3-L1 adipocytes

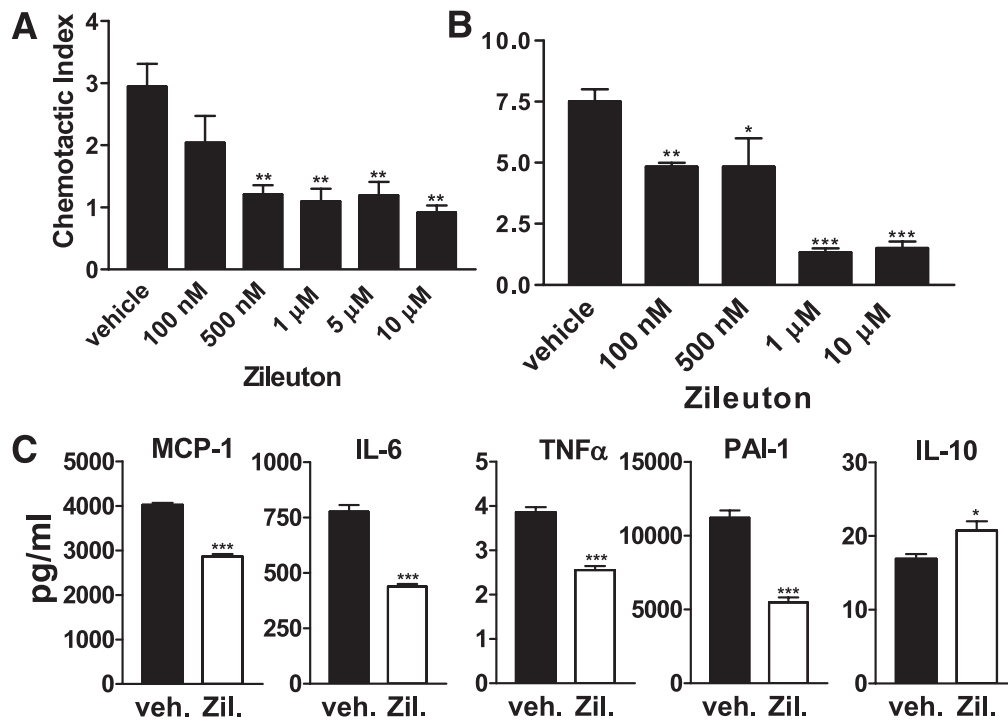


FIG. 2. Role of LTs in adipocyte-induced RAW264.7 macrophage chemotaxis and adipokine secretion. The chemotactic index or ratio of RAW264.7 macrophages (A) or Jurkat T cells (B) in the lower chamber of an 8-μm filter insert in the presence vs. the absence (serum-free DMEM) of chemoattractant was determined using CM from 3T3-L1 adipocytes that were treated with increasing amounts of the 5-LO inhibitor Zileuton (Zil.). C: Secretion in pg/mL of MCP-1, IL-6, TNF-α, PAI-1, and IL-10 by 3T3-L1 adipocytes treated with Zileuton (5 μmol/L) or vehicle (veh.). Data are expressed as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001 compared with vehicle control (1% DMSO).

treated or not with increasing concentrations of the 5-LO inhibitor Zileuton. As shown in Fig. 2A, CM harvested from vehicle-treated 3T3-L1 adipocytes induced chemotaxis of the RAW264.7 macrophages, whereas CM from Zileuton-treated 3T3-L1 adipocytes exhibited a concentration-dependent decrease in chemotactic potential with full blockage of chemotaxis at 500 nmol/L Zileuton. Similarly, Jurkat T-cell chemotaxis was fully blocked at 1 μmol/L Zileuton (Fig. 2B). From this, we conclude that inhibiting LT production by adipocytes can fully block their chemotactic potential for macrophages and T cells.

We also studied the effect of Zileuton treatment on cytokine/chemokine secretion by 3T3-L1 adipocytes. Figure 2C shows that the treatment resulted in reduced secretion of the proinflammatory cytokines/chemokines monocyte chemoattractant protein 1 (MCP-1), interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), and plasminogen activator inhibitor 1 (PAI-1), and increased secretion of the anti-inflammatory cytokine IL-10. Together, these in vitro results suggest that LTs produced by adipocytes can play an important role in the macrophage and T-cell infiltration and increased expression of inflammatory cytokines/chemokines observed in obese AT.

Metabolic analyses of HFD-fed mice deficient for 5-LO or treated with the 5-LO inhibitor Zileuton. To investigate the in vivo role of LTs in obesity-associated inflammation and IR, we used LT-deficient 5-LO^{-/-} mice. These mice and their WT counterparts were fed NC or a HFD for 15 weeks. HFD-induced weight gain was more pronounced in 5-LO^{-/-} mice compared with their WT counterparts (Table 1 and Fig. 3A). This increased weight gain observed in the 5-LO^{-/-} mice seems to be due to increased food intake (Fig. 3B). The ITT and GTT results showed that the 5-LO^{-/-} mice were partially protected against

HFD-induced IR compared with their WT counterparts (Fig. 3C). This was observed in animals matched for weight and diet-duration, indicating that this protective phenotype persists despite the increased weight gain observed in the 5-LO^{-/-} animals (see Table 2 for weights during metabolic testing). The ITT results indicate that even when fed NC, the 5-LO^{-/-} mice are more insulin-sensitive than the WT mice. Serum insulin levels measured during the GTTs show that the 5-LO^{-/-} mice need to secrete less insulin to normalize their blood glucose levels compared with their WT counterparts, regardless of their diet (Fig. 3E). The latter confirms that LT-deficiency renders mice more insulin-sensitive than their WT counterparts. In agreement with this phenotype, fasting serum glucose and insulin levels are also lower in HFD 5-LO^{-/-} mice compared with their WT counterparts (Table 1).

Similarly, HFD-fed mice that were already IR regained insulin sensitivity after 2 weeks of daily treatment with the 5-LO inhibitor Zileuton, as measured by ITTs and GTTs (Fig. 3D). Although the GTT shows two overlapping curves, the serum insulin measurements (Fig. 3F) indicate that less insulin was needed in the Zileuton-treated mice compared with the vehicle-treated animals to obtain the same blood glucose-lowering response. In agreement with this, fasting serum insulin levels were lower in the Zileuton-treated compared with vehicle-treated animals (Table 1). The Zileuton treatment reduced the epi-WAT LTB4 and CysLT levels by 74% and 65%, respectively (data not shown). Together, these in vivo experiments demonstrate that the absence of LTs and the inhibition of their production both lead to a protection against HFD-induced IR in mice.

ATM and T-cell quantification in HFD-fed mice deficient for 5-LO or treated with the 5-LO inhibitor Zileuton. Next, we quantified macrophages and T cells in AT from

TABLE 1
Body and tissue weights and serum characteristics of animals studied

	NC		HFD		HFD	
	WT	5-LO ^{-/-}	WT	5-LO ^{-/-}	WT vehicle	WT Zileuton
Body weight (g)	33.80 ± 0.47	34.33 ± 1.49	49.55 ± 1.18	53.14 ± 0.77*	49.03 ± 0.32	48.3 ± 1.54
Sc-WAT (g)	0.67 ± 0.08	0.50 ± 0.09	2.70 ± 0.13	2.71 ± 0.08	2.79 ± 0.10	2.73 ± 0.22
Epi-WAT (g)	0.95 ± 0.18	1.03 ± 0.20	1.53 ± 0.14	1.70 ± 0.12	1.60 ± 0.06	1.57 ± 0.08
Liver (g)	1.18 ± 0.04	1.31 ± 0.20	2.24 ± 0.15	2.01 ± 0.22	2.10 ± 0.08	2.26 ± 0.18
Serum glucose (mg/dL)	184.7 ± 4.1	169.5 ± 7.4	252.9 ± 12.6	214.8 ± 8.4*	229.2 ± 17.0	224.7 ± 15.1
Serum insulin (ng/mL)	1.07 ± 0.09	0.89 ± 0.10	5.49 ± 0.70	3.57 ± 0.36*	6.22 ± 0.82	1.88 ± 0.32**

Data are expressed as mean ± SEM. **P* < 0.05, ***P* < 0.01 indicating significant difference between genotype/treatment group within diet (*n* ≥ 6/group).

HFD-fed 5-LO^{-/-} mice or their WT counterparts treated with the 5-LO inhibitor Zileuton. Immunohistochemical stainings for the macrophage marker MAC2 or the T-cell marker CD3 both show a reduction of crown-like structures in AT from HFD-fed 5-LO^{-/-} and Zileuton-treated WT mice compared with respective controls (Fig. 4A and B). This reduction of ATMs and T cells was confirmed by qPCR analysis of the relative mRNA levels of the macrophage marker CD68 and T-cell marker CD3, although the decrease observed with Zileuton treatment did not reach statistical significance for CD68 (Fig. 4C and E). FACS analysis of the SVCs isolated from AT shows that the decrease in ATMs is largely due to a reduction in proinflammatory M1 (F4/80⁺CD11b⁺CD11c⁺) macrophages with a minor but not significant decrease in number of anti-inflammatory M2 macrophages (F4/80⁺CD11b⁺CD11c⁻) (Fig. 4D). However, the proportion of M2 macrophages as a percentage of total macrophages actually increased (Fig. 4D), suggesting that LT absence leads to a shift in proportion of M1 versus M2 macrophages.

A similar FACS analysis demonstrated that there is a global reduction in T cells with an equal relative reduction of both CD4⁺ and CD8⁺ T cells without a shift in percentages of either T cell subset (Fig. 4F). Together, these results demonstrate that the absence of LTs and the inhibition of their production both lead to a reduction of ATMs and T cells, with a preferential decrease in the proinflammatory M1 macrophage subset.

Serum protein and AT mRNA levels of pro- and anti-inflammatory cytokines/chemokines. Finally, we measured serum protein and AT mRNA levels of pro- and anti-inflammatory cytokines/chemokines in HFD-fed mice deficient for 5-LO or treated with the 5-LO inhibitor Zileuton. The absence of LTs and the inhibition of their production both leads to a decrease in serum protein levels for the proinflammatory cytokines/chemokines MCP-1, IL-6, TNF- α , and PAI-1 and an increase in the anti-inflammatory cytokine IL-10 (Fig. 5A). Similar results were obtained when we measured the relative mRNA levels of these cytokines/chemokines in AT (Fig. 5B). Together, these findings show that LT deficiency results in an improved inflammatory state in HFD-fed mice, both systemically and locally in AT.

DISCUSSION

The current study provides novel information on the capacity of mouse and human adipocytes to produce LTs. Furthermore, we show that adipocyte LT production increases with obesity and positively correlates with adipocyte size. Our chemotaxis experiments suggest that LTs play a crucial role in macrophage and T-cell chemotaxis induced by 3T3-L1 CM

(Fig. 2). The complete block of macrophage and T-cell chemotaxis with Zileuton treatment could be explained by indirect effects of blocking of LT synthesis on the production of other chemotactic factors such as MCP-1. Previous work showed that LTs can induce MCP-1 expression in macrophages (20), and we now demonstrate that Zileuton treatment of 3T3-L1 adipocytes leads to a reduced secretion of MCP-1 by these cells (Fig. 2C), suggesting that LTs produced by adipocytes can stimulate MCP-1 production by those cells in an autocrine fashion.

Our in vivo results show some discrepancies with earlier publications. A recent publication demonstrated an increase in LTB4 levels in AT from obese *ob/ob* mice compared with lean mice but did not observe a change in CysLT levels (12). Although we used a diet-induced model of obesity, this other study investigated the leptin-deficient *ob/ob* mice. Leptin has been shown to induce LT production (21,22), so the leptin deficiency might explain the absence of the increase of CysLT levels in the AT of these mice. Furthermore, although the same study showed a decrease in TNF- α and IL-6 mRNA levels in AT and 3T3-L1 adipocytes treated with a FLAP inhibitor, similar to our observations when using a 5-LO inhibitor, these investigators did not see a decrease in MCP-1 mRNA levels in AT and only a slight, nonsignificant, decrease in 3T3-L1 adipocytes treated with the FLAP inhibitor. Their MCP-1 results are somewhat surprising considering that they show in the same study that LT treatment of AT explants results in a dose-dependent increase in MCP-1 secretion.

Another group published that 5-LO^{-/-} mice are more prone to weight gain even when fed a NC diet and that this is not due to increased food intake (23). We also observed that the 5-LO^{-/-} mice gained more weight than their WT counterparts, but this only reached statistical significance when the animals were fed a HFD (Table 1, Fig. 3A). Despite the increased weight of the HFD-fed 5-LO^{-/-} mice, we did not observe significant increases in fat pad weight. We believe that the weight change was too small to monitor with significant changes in individual fat pad weight. However, we did observe a difference in food intake in our animals, regardless of diet (Fig. 3B). Perhaps the other investigators missed the difference in food intake because they only measured daily food intake for a 6-day period, whereas we measured daily food intake throughout the 13 weeks of diet. Furthermore, these investigators published another study claiming that 5-LO^{-/-} mice are hyperglycemic and that they exhibit impaired insulin secretion in response to a bolus glucose injection, resulting in significantly higher plasma glucose levels than WT mice (24). We actually detected lower fasting plasma glucose levels in 5-LO^{-/-} compared with WT mice, although this difference was not

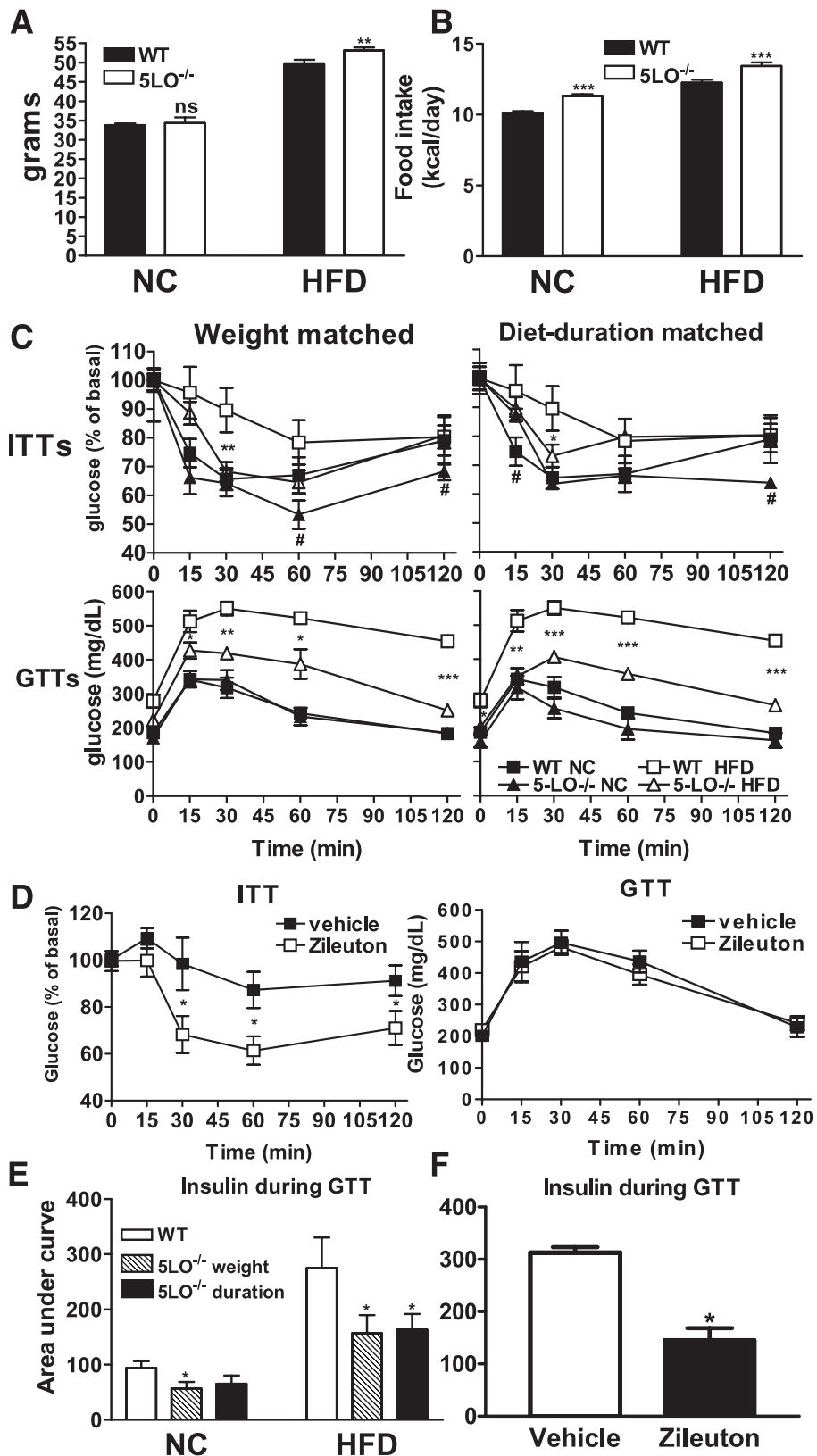


FIG. 3. Metabolic analyses of HFD-fed mice deficient for 5-LO or treated with the 5-LO inhibitor Zileuton. **A:** Weight in grams of WT and 5-LO^{-/-} mice after 15 weeks of NC or HFD feeding. **B:** Food intake in kcal/day of WT and 5-LO^{-/-} mice fed NC or HFD. ****P** < 0.01, *****P** < 0.001 compared with WT counterpart. **C:** ITTs and GTTs in weight- or diet duration-matched WT and 5-LO^{-/-} mice fed NC or HFD. **#P** < 0.05 comparing WT NC with 5-LO^{-/-} NC. ***P** < 0.05, ****P** < 0.01, and *****P** < 0.001 comparing WT HFD with 5-LO^{-/-} HFD. **D:** ITT and GTT comparing WT HFD mice that received a daily oral treatment for 2 weeks with vehicle (10% DMSO) or Zileuton (100 mg/kg). Plasma insulin levels during the GTTs are presented as area under curve for weight- or diet duration-matched WT and 5-LO^{-/-} mice fed NC or HFD (**E**) or when comparing Zileuton- with vehicle-treated mice (**F**). ***P** < 0.05 when comparing with WT or vehicle-treated mice. Data are expressed as mean ± SEM (*n* ≥ 6/group).

TABLE 2
Body weights during metabolic tests

	WT		Weight-matched 5-LO ^{-/-}		Diet duration-matched 5-LO ^{-/-}	
	NC	HFD	NC	HFD	NC	HFD
ITT						
Weeks of diet	9	9	7	7	9	9
Average BW (g)	29.63 ± 0.35	44.00 ± 1.29	31.00 ± 0.58	43.00 ± 1.48	30.50 ± 0.87	46.20 ± 1.28*
GTT						
Weeks of diet	13	13	10	10	13	13
Average BW (g)	30.86 ± 0.75	48.26 ± 0.75	30.67 ± 0.33	47.40 ± 1.47	32.75 ± 0.85	52.40 ± 0.81*

Data are expressed as mean ± SEM. BW, body weight. **P* < 0.05 between genotype within diet (*n* ≥ 6/group).

statistically significant in NC-fed mice (Table 1). In addition, we observed a decrease in plasma glucose levels in HFD-fed 5LO^{-/-} mice compared with their WT counterparts after a bolus glucose injection (Fig. 3C). Although we do find a decrease in fasting plasma insulin levels and glucose-induced secretion of insulin during GTTs in both 5-LO^{-/-} and HFD-fed Zileuton-treated mice, we believe that this simply reflects the increased insulin-sensitive state of these mice. In support of our findings, studies performed on apolipoprotein E^{-/-}/5-LO^{-/-} double-knockout mice also showed improved insulin sensitivity and reduced serum glucose levels compared with apolipoprotein^{-/-} mice, confirming that 5-LO deficiency leads to increased insulin sensitivity (25). Nonetheless, our data cannot rule out decreases in insulin secretion in the 5-LO^{-/-} and Zileuton-treated mice that may exhibit better glucose tolerance

through compensation by increased insulin sensitivity due to decreased inflammation.

We observed differences in ATM and T-cell content between both of our control groups, with more macrophages and fewer T cells present in the AT from the vehicle-treated HFD-fed WT control group compared with the HFD-fed WT mice used as a control group for the 5-LO^{-/-} mice (Fig. 4). These differences were most likely because these mice had been fed the HFD for different intervals (15 weeks for the 5-LO^{-/-} study and 17 weeks for the Zileuton study). Regardless of these differences between the control groups, the reduction in both ATM and T-cell content is similar whether LTs are absent throughout the development of obesity (5-LO^{-/-}), or LT production is blocked once obesity has been established (Zileuton treatment of HFD-fed mice). We show that this reduction of inflammatory cells in AT is

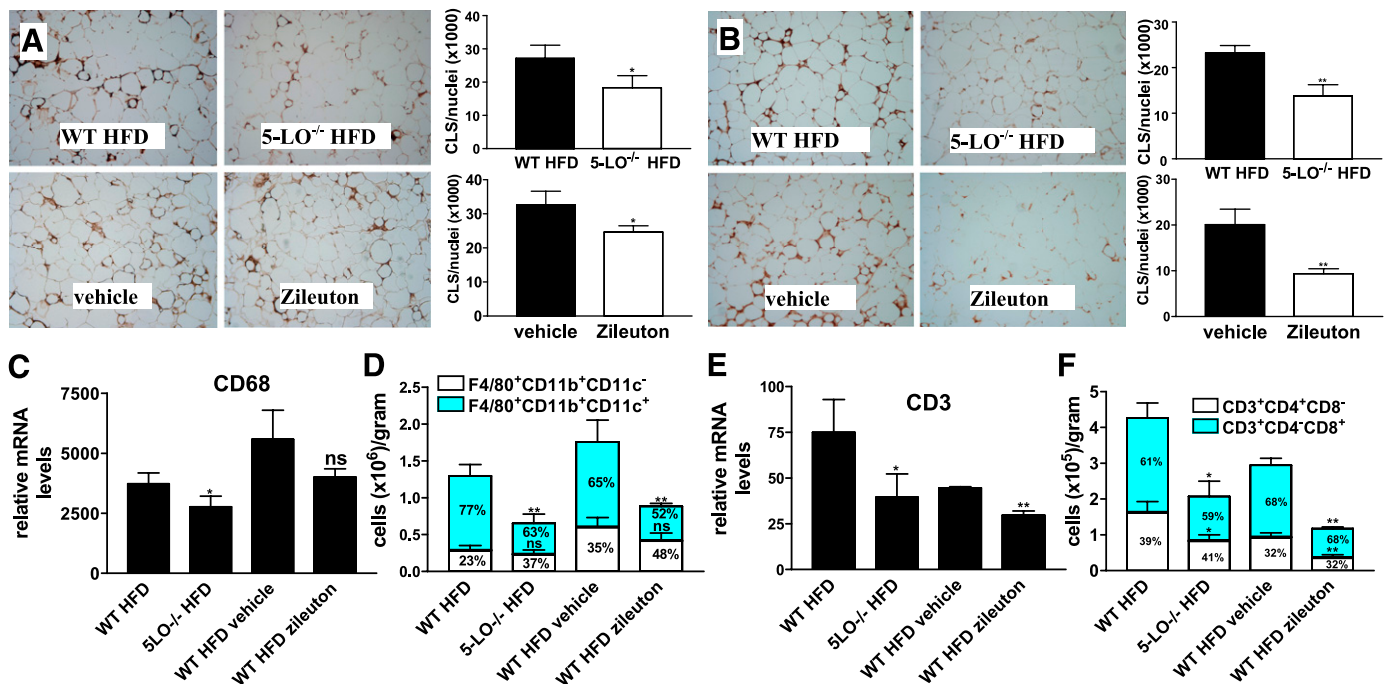


FIG. 4. ATM and T-cell quantification in HFD-fed mice deficient for 5-LO or treated with the 5-LO inhibitor Zileuton. Immunohistochemical stainings for the macrophage marker MAC2 (A) or the T-cell marker CD3 (B) in paraffin-embedded epi-WAT sections from HFD-fed WT and 5-LO^{-/-} mice and HFD-fed WT mice treated with vehicle or Zileuton. Quantitative representations of the illustrations on the left are shown in the graphs on the right of each panel, where ATM or T-cell presence is presented as crown-like structures (CLS) per 1,000 nuclei. qPCR analysis of relative mRNA levels of the macrophage marker CD68 (C) or the T-cell marker CD3 (E) in the same set of epi-WAT tissues. FACS analysis of SVCs derived from again the same samples, quantifying the total number of macrophages (D) or T cells (F) per gram epi-WAT and their composition in percentages of M2 (F4/80⁺CD11b⁺CD11c⁻) and M1 (F4/80⁺CD11b⁺CD11c⁺) ATMs or CD4⁺ (CD3⁺CD4⁺CD8⁻) and CD8⁺ (CD3⁺CD4⁺CD8⁺) T cells, respectively. Data are expressed as mean ± SEM (*n* ≥ 6/group). **P* < 0.05 and ***P* < 0.01 compared with WT or vehicle control groups. (A high-quality digital representation of this figure is available in the online issue.)

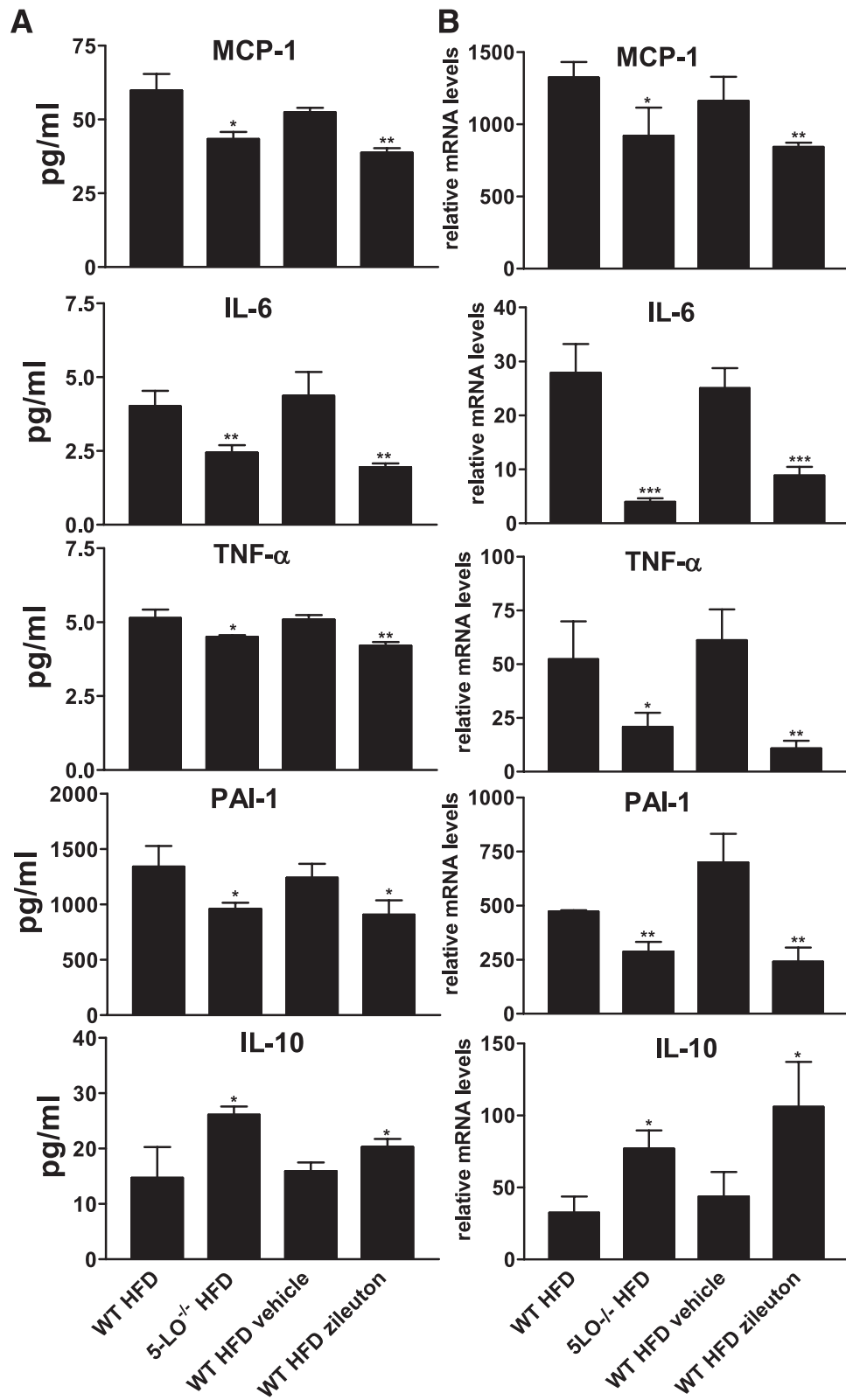


FIG. 5. Serum protein and AT mRNA levels of pro- and anti-inflammatory cytokine/chemokines. *A:* Fasting serum levels of MCP-1, IL-6, TNF α , PAI-1, and IL-10 in HFD-fed WT and 5-LO^{-/-} mice and HFD-fed WT mice treated with vehicle or Zileuton. *B:* Relative mRNA levels measured by qPCR of the same set of pro- and anti-inflammatory cytokines/chemokines in epi-WAT from the same groups of mice. Data are expressed as mean \pm SEM ($n \geq 6$ /group). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with WT or vehicle control groups.

accompanied by a reduction in AT mRNA levels of proinflammatory factors (Fig. 5B). Our data suggest that the latter reduction is not solely due to a decrease in inflammatory cell infiltration of AT because we demonstrate that inhibiting LT production by adipocytes leads to a reduction of secretion of proinflammatory factors by the same cells. Our studies do not allow us to draw conclusions regarding the relative roles of LT production by the adipocyte and stromal vascular compartments in the phenotypes observed.

Zileuton is currently used under the trade name ZYFLO for the maintenance treatment of asthma. Interestingly, there is a growing research interest that focuses on the association between asthma and obesity, and a possible mechanism linking both concerns immunoregulation and inflammation (26). Moreover, a recent study suggests that asthma in obese patients is LT-driven (27). It would be of interest to perform prospective studies to determine if obese asthmatic individuals do indeed demonstrate superior therapeutic responses to LT-modifying agents and whether the same patients also show improvements in insulin sensitivity as a result of their treatment.

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No potential conflicts of interest relevant to this article were reported.

I.M.-S. and P.A.G. researched the data and reviewed and edited the manuscript. C.F., H.A., C.P., V.B., and J.G., researched the data. C.C.F., A.B., and E.V.O. reviewed and edited the manuscript. J.G.N. researched the data and wrote the manuscript. J.G.N. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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